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Exploring the biology of vascular calcification in chronic kidney disease: What's circulating?

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Chronic kidney disease (CKD) is associated with fatal cardiovascular consequences in part due to ectopic calcification of soft tissues particularly arteries, capillaries, and cardiac valves. An increasing body of evidence from experimental studies and *in vivo* data suggest that (I) a mineral imbalance with hyperphosphatemia and high-circulating calcium × phosphate product, (II) a deficiency of systemic or local calcification inhibitors, (III) death or 'damage' of vascular smooth muscle cells (VSMCs), and/or (IV) phenotypic transformation of VSMCs to osteo/chondrocytic cells may all act in concert to initiate and sustain vascular calcification. In CKD patients inhibitory systems are overwhelmed by a multitude of agents that induce VSMC damage and cell death resulting in the release of vesicles capable of nucleating basic calcium phosphate. Studies with genetically altered mice have identified both local and systemic calcification inhibitors that act to maintain VSMC differentiation or regulate vesicle properties. However, for many of these proteins the mechanisms and sites of action are still under investigation. In particular, it is unclear whether factors present in the circulation have an inhibitory role there and whether circulating levels of these proteins influence or are indicative of underlying disease processes in individual patients. A greater understanding of the origins and roles of potential circulating inhibitors may result in novel strategies aimed at the prevention or reversal of the life-limiting calcifying vasculopathies seen in CKD patients.

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Cardiovascular disease is the most common cause of death in patients with chronic kidney disease (CKD) with a 30-fold higher mortality than the general population despite adjustment for traditional cardiovascular risk factors such as diabetes mellitus and hypertension.[1] Recent epidemiological studies have shown that even minimal alterations in renal function (as evidenced by a reduced glomerular filtration rate, presence of microalbuminuria, and elevated serum phosphate levels in the general population) pose potent cardiovascular risks.[2,3] Vascular calcification is a significant contributor to cardiovascular risk in CKD patients, and its extent and severity has been correlated with mortality in several studies.[4]

Calcification of arteries occurs in the intima in association with atherosclerosis, where it may contribute to plaque formation and rupture, and in the media, where it causes vascular stiffening. Although a combination of intimal and medial calcification may occur in patients with CKD, either process may occur independently of the other, and, at least in adolescents and young adults with CKD, the vascular 'calcium load' is almost exclusively medial. Increased arterial stiffness is mechanistically linked with systolic hypertension, left ventricular hypertrophy, and reduced coronary perfusion, and is a significant independent predictor of mortality.[5] Direct evidence of the calcification burden is obtained from plain X-ray or, in recent years, from cardiac computed tomography (CT) scans (electron beam CT or helical/multislice CT). However, neither can differentiate between medial and intimal calcification and, as both tests measure overt calcification only, they are not sensitive enough to measure early vascular calcium load. The ability to identify and quantitate early vascular calcification in patients initiating dialysis may allow us to predict their likelihood of developing accelerated calcification and would have implications for their treatment regime. This has become particularly relevant as the 4D study has shown that statins are ineffective in reducing cardiovascular events in diabetic patients with CKD, and the availability of non-calcium-based phosphate binders like sevelamer and lanthanum carbonate can allow for treatments that are less likely to contribute to the calcium load for patients susceptible to progression of calcification. As an alternative to the currently available imaging methods,

the use of circulating biomarkers may be a useful and convenient measure of an individual patient's susceptibility to calcify. Evidence is accumulating to suggest that relevant biomarkers might include a subset of circulating proteins that have been shown to act as inhibitors of vascular smooth muscle cell (VSMC) calcification.

VASCULAR CALCIFICATION IS A HIGHLY REGULATED PROCESS

Vascular calcification was previously thought to be a passive process caused by precipitation of mineral from the circulation, particularly in patients with a mineral imbalance. However, as early as the 19th century, Virchow described bone-like structures in the vasculature, which provided the first clue that vascular calcification may be a regulated process. More recent studies have described true bone marrow, osteo/chondrocytic cells, cytokines, transcription factors, matrix proteins, and matrix vesicles characteristic of mature osteoblasts and terminally differentiated chondrocytes within calcified lesions, with the inorganic material being hydroxyapatite, the same mineral found in physiologic biomineralization.[4] Local VSMCs as well as circulating mesenchymal stem cells, local pericytes, and fibroblasts that exhibit multilineage potential may transdifferentiate into osteo/chondrocytic cells in the arterial wall and orchestrate bone formation and calcification. The osteo/chondrocytic conversion of VSMCs and stem cells both *in vitro* and *in vivo* is accompanied by upregulation of Cbfa1/Runx2, osterix, Msx2, and Sox9 transcription factors that are centrally involved in chondrocyte maturation and osteoblastic differentiation. Accumulating evidence suggests that multiple factors such as hypertension, reactive oxygen species, advanced glycation end products, lipids, inflammatory proteins, such as tumor necrosis factor-α, and potentially other, as yet unidentified, damage-inducing agents initiate osteo/chondrocytic conversion and matrix vesicle release in VSMCs. However, in CKD, a mineral imbalance is central to this damage with elevated phosphate inducing expression of Runx2 and osterix in VSMCs. More significantly, apoptotic bodies and matrix vesicles similar to those that nucleate mineral in bone are released from dying and damaged VSMCs and nucleate mineral in vascular tissues.[6] Elevated calcium induces VSMC death and increases matrix vesicle release, whereas calcium and phosphate both increase the mineralization potential of the released matrix vesicles. Finally, in concert with this phenotypic change and damage, VSMCs lose expression of endogenous mineralization inhibitors, further accelerating their osteo/chondrocytic conversion as well as reducing their capacity to limit the mineralization process (Figure 1).

Animal models with targeted disruption of genes as well as *in vitro* studies have been central in identifying naturally occurring inhibitors of calcification (Table 1). Fetuin-A (α_2-Heremans-Schmid glycoprotein)[7] is a serum protein produced in the liver that exerts its inhibitory effects via the circulation. However, many proteins such as matrix Gla protein (MGP),[8] Smad6,[13] nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1),[12] Klotho,[10] and osteoprotegerin (OPG)[9] are expressed locally by VSMCs in the healthy vessel wall. Interestingly, a number of these inhibitors are concentrated in VSMC-derived matrix vesicles where they act to block mineral nucleation. Moreover, they also find their way into the circulation where they can be measured, suggesting that they may have actions both locally and within the circulation. In the remainder of this review, we explore the evidence that inhibitory proteins function within the circulation, and the possibility that their circulating levels may inform us of underlying disease processes.

CIRCULATING INHIBITORS

Fetuin-A

All extracellular fluid contains calcium and phosphate in concentrations exceeding their solubility product for spontaneous precipitation, suggesting that under normal conditions protein inhibitors of ectopic soft tissue calcification prevent the development or progression of vascular calcification. *In vitro* studies have shown that, whereas serum from normal subjects is a potent inhibitor of VSMC calcification, serum from CKD patients lacks this protective effect.[6] Although this might be due to the presence of toxins in uremic serum, an alternate explanation may be that serum from dialysis patients lacks inhibitors, and fetuin-A is a paradigm for this model. Fetuin-A belongs to the cystatin superfamily of proteins, is made predominantly by the liver, and acts as a negative acute phase reactant; its production is therefore downregulated in systemic inflammation. *Fetuin-A*$^{-/-}$ mice exhibit mild ectopic calcification, but extensive mineralization can be induced in almost all soft tissues when crossed onto a DBA/2 calcification-susceptible mouse strain or when these mice are fed on a mineral- and vitamin D-rich diet or on high-fat diet.[7] Fetuin-A acts systemically by binding excess mineral and inhibiting basic calcium phosphate precipitation in serum and extracellular fluids,[7] and is also found in the circulation of rats complexed to bone-derived hydroxyapatite (the 'fetuin-mineral complex'), which may represent another protective mechanism to limit deposition of mineral in the vasculature.[14] Importantly, fetuin-A is a multifunctional protein that in addition to its systemic effects can also modulate the calcification processes locally. At sites of vascular damage it is taken up by VSMCs, incorporated into intracellular vesicles, and then released within matrix vesicles where it potently inhibits mineral nucleation. In addition, fetuin-A inhibits VSMC apoptosis and aids in phagocytosis of extracellular vesicles, thus further limiting mineralization.[15]

In vivo, systemic fetuin-A levels are significantly lower in hemodialysis patients than in healthy controls, possibly as a result of low-grade inflammation induced by dialysis.[16] Moreover, polymorphisms in the fetuin-A gene correlate with circulating levels of phosphate, suggesting that genetic variants resulting in changes in fetuin-A protein function may predispose some patients to calcification.[17] However, fetuin-A only partially accounts for some of the capacity of

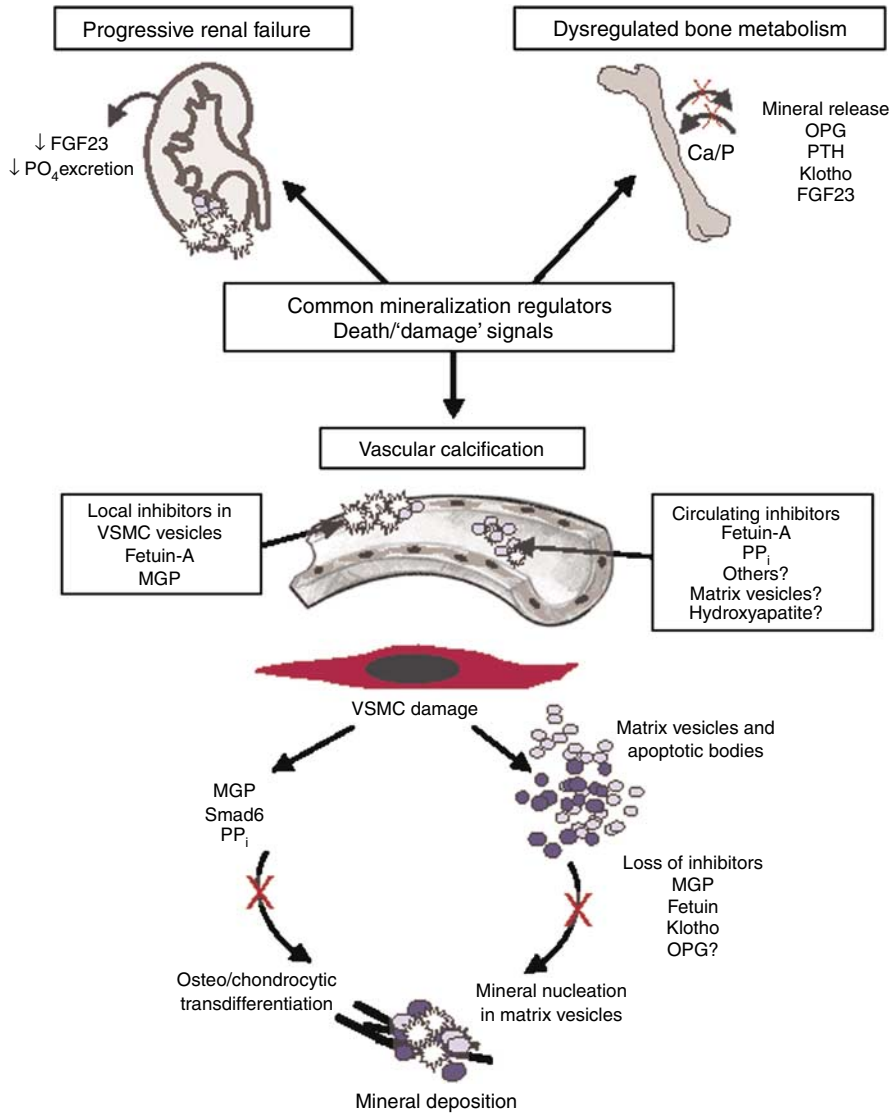

Figure 1 | Soft tissue calcification, vascular calcification, and bone loss are common features of the pathology in CKD patients. These processes are driven by common mechanisms of damage, vesicle release, and loss of mineralization regulating proteins both locally and systemically. An understanding of how these processes are related is just beginning to emerge. The origins, effects, and potential of circulating mineralization regulators to act as biomarkers of disease are key areas for further investigation. Ca, calcium; FGF23, fibroblast growth factor-23; MGP, matrix Gla protein; NPP1, nucleotide pyrophosphatase-1; OPG, osteoprotegerin; P, phosphate; PP_i, inorganic pyrophosphate; PTH, parathyroid hormone; VSMC, vascular smooth muscle cell.

serum to inhibit calcification, suggesting that other as yet unidentified serum proteins can also inhibit calcification. Although some of these, like the closely related protein fetuin-B and albumin, are likely to be serum proteins not previously considered as regulators of calcification, other candidates include calcification inhibitors produced in soft tissues or bone that are also present in the circulation (Table 2). Some proteins such as MGP have been identified as a component of the 'fetuin-mineral complex' described in rats and are also concentrated in kidney stones suggesting that they can be absorbed from serum and then bind to calcified structures. However, as discussed below, evidence that these proteins have a regulatory role in serum remains controversial.

MGP

MGP, a low-molecular-weight protein found in bone, cartilage, and highly expressed in kidney, cardiac valves, and the media of arteries, contains five residues of the vitamin K-dependent amino acid γ-carboxyglutamic acid (Gla). Warfarin, commonly used in hemodialysis patients, inhibits the vitamin-K-dependent γ-carboxylation of MGP and is potentially a risk factor for the development of calciphylaxis. $MGP^{-/-}$ mice have inappropriate calcification of cartilage and die within weeks after birth due to rupture of their heavily calcified aorta.[8] Within calcified vessels, cartilaginous metaplasia occurs, which may be related to the ability of MGP to bind bone morphogenetic protein-2 (BMP2): lack of MGP leads to increased BMP2 activity, which is likely to

Table 1 | Gene disruption studies in mice with a phenotype of vascular calcification

Gene	Vascular phenotype, affected vessels	Present in VSMC vesicles
Fetuin-A (α_2-Heremans-Schmid glycoprotein)	Ectopic calcification of small blood vessels, most organs (e.g., myocardium, lung, kidney, skin)[a7]	Yes
Matrix Gla protein (MGP)	Medial calcification of arteries, aortic valves (not arterioles, capillaries, or veins), cartilaginous metaplasia within the vessel wall[8]	Yes
Osteoprotegerin (OPG)	Medial and subintimal calcification of the aorta and renal arteries, presence of multinuclear osteoclast-like cells within the vascular wall[9]	[c]
Klotho	All calibers of arteries affected, intimal thickening of medium-sized arteries[10]	[c]
Fibroblast growth factor-23 (FGF23)	Vascular calcification in kidneys[11]	[c]
Nucleotide pyrophosphatase/ phosphodiesterase-1 (NPP1)	Aortic medial calcification, intraaortic cartilaginous differentiation of VSMCs[12]	[c]
Madh6[b]	Cartilaginous metaplasia and ossification of the media of larger vessels, hyperplasia of cardiac valves[13]	[c]

VSMC, vascular smooth muscle cell.
[a]On a DBA/2 genetic background or a mineral/vitamin D-rich diet.
[b]Encodes Smad6 protein.
[c]Presence in VSMC-derived vesicles not tested.

induce VSMC chondrocytic conversion. MGP is also found in matrix vesicles, wherein it acts like fetuin-A to limit mineral nucleation, and it is also present in serum.[6] However, transgenic studies have shown that rescue of calcification only occurs if MGP is expressed in VSMCs, not if it is only present systemically.[33] Thus, the role of MGP in the circulation, if any, remains largely unknown, and although MGP gene polymorphisms may be a prognostic factor for the progression of cardiovascular disease in CKD patients,[22] to date MGP has not proved to be a good circulating marker of calcification. Although some studies have shown that serum MGP is a risk factor for atherosclerosis, associations between circulating MGP levels and coronary artery calcification have given conflicting results.[34,35] However, it may be that we are measuring the 'wrong' MGP. A recent study has shown that in healthy arteries MGP deposition was associated with elastic fibres in the tunica media, with no undercarboxylated MGP present. In contrast, in vessels with intimal and/or medial calcification, undercarboxylated MGP was localized around all areas of calcification, suggesting that impaired carboxylation of MGP, potentially due to warfarin use or low vitamin K status, is associated with intimal and medial vascular calcification.[20] Moreover, the incorporation of undercarboxylated MGP in matrix vesicles increases their capacity to calcify. Thus, circulating MGP levels may indeed be a biomarker of VSMC damage and calcification, but it may be necessary to measure undercarboxylated MGP serum levels, and these have not been assessed so far.

OPG

OPG is a soluble decoy receptor for the principal regulator of osteoclasts, receptor activator of nuclear factor-κB ligand (RANKL), which stimulates all aspects of osteoclast biology including differentiation, activation, fusion, and survival. By blocking RANKL, OPG inhibits bone resorption, but OPG is also expressed in a variety of cells and tissues, especially in the media of arteries. Targeted deletion of the OPG gene in mice resulted not only in osteoporosis, but also in calcification of the great arteries (aorta, renal arteries) without vascular challenge. Calcification in these mice could be rescued by introduction of an *opg* transgene from mid-gestation but not by parenteral application of OPG after mineralized lesions were established; this is in contrast to osteoporosis, which could be efficiently treated by a parenteral OPG regimen.[9] Thus, although the precise role of OPG in the vascular wall and its possible interaction with VSMCs has yet to be determined, like MGP it does not appear to function if present only systemically. Interestingly, although OPG is deposited at sites of calcification and globally downregulated in the diseased vasculature, circulating OPG levels are increased in patients on hemodialysis, and have been linked to the presence and extent of coronary artery disease.[9] Given that OPG is produced by a variety of cell types, the source of elevated OPG remains elusive and it is unclear whether increased systemic OPG levels reflect the cause or consequence of vascular calcification or are not associated at all. It is interesting to speculate that circulating OPG may in itself be a damaging agent for VSMCs in a manner analogous to glucose in diabetes and PTH in CKD. Clearly, further studies are required to determine the effects of elevated OPG on VSMC function.

Premature aging-related genes: Klotho and FGF23

These two multifunctional proteins regulate calcium phosphate metabolism as well as aging, and deletion studies have shown that they play a role in vascular calcification. Klotho is a pleiotrophic transmembrane protein that is thought to repress intracellular signaling of insulin and insulin-like growth factor-1 (IGF1). It also acts as a cofactor for the fibroblast growth factor (FGF) receptor 1c in FGF23 signaling in what appears to be an evolutionarily conserved mechanism acting to suppress aging. Recent studies have shown that *Klotho*- and *FGF23*-deficient mice show similar phenotypes, including hyperphosphatemia and hypercalcemia, in addition

Table 2 | Calcification regulators identified in the circulation (separate document)

Factor	Role in circulation	Levels in circulation	Human single gene defects or genetic polymorphisms
Fetuin-A (α_2-Heremans-Schmid glycoprotein)	Inhibitor	Reduced in CKD[16]	Polymorphisms may predispose patients to vascular calcification[18]
Albumin	Inhibitor[a]	Low serum albumin in CKD, predicts cardiovascular events and morbidity[19]	[a]
Matrix Gla protein (MGP)	No effect[b]	Conflicting results for serum levels in vascular calcification[20]	Keutel syndrome-extensive vascular calcification and abnormal calcification of cartilage[21] MGP gene polymorphisms may be prognostic for vascular calcification[22]
Osteoprotegerin (OPG)	[a]	Increased in CKD and patients with vascular calcification[9]	Juvenile Paget's disease-an autosomal-recessive osteopathy, but no clear association with vascular disease[9] Polymorphisms in the promoter region of OPG are associated with atherosclerosis[9]
Receptor activator of NF-κB ligand (RANKL)	Inducer[a]	Increased in CKD[23] and serum levels may be a predictor of vascular risk	[a]
Klotho	[a]	Decline with age[a]	Polymorphism may be a genetic risk factor for coronary artery disease[24]
Fibroblast growth factor-23 (FGF23)	[a]	Increased in CKD[25]	[a]
Inorganic pyrophosphate (PP_i)	Inhibitor[a]	Reduced in CKD, and also removed by hemodialysis[26]	Infantile idiopathic arterial calcification—calcification of the internal elastic laminae of large vessels, often with death in the first year of life[4]
Bone morphogenetic protein-2 (BMP2)	Inducer[a]	Increased in CKD[a]	[a]
Bone morphogenetic protein-7 (BMP7)	Inhibitor[a]	[a]	[a]
Osteopontin (OPN)	Inhibitor[a]	Increased in CKD,[27] conflicting results for serum levels in vascular calcification	Sequence variation associated with carotid intima-media thickness[28]
Osteocalcin (OC)	No effect[b]	No clear correlation between glomerular filtration rate and serum osteocalcin, lower serum levels in vascular disease[29]	[a]
Bone-specific alkaline phosphatase	[a]	Increased in CKD[23] and vascular calcification[30]	[a]
Mineral and hormonal regulators			
Calcium (Ca)	Inducer	Increased in CKD, risk factor for cardiovascular events in dialyzed patients[1]	[a]
Phosphate (P)	Inducer	Increased in CKD and vascular calcification[3]	[a]
Magnesium (Mg)	Inhibitor	Inverse relationship between serum magnesium and vascular calcification in CKD[31]	[a]
Parathyroid hormone (PTH)	Inducer/inhibitor	Increases Ca absorption and influences bone turnover,[1] direct effect on VSMCs[a]	[a]
Vitamin D	Inducer/inhibitor	Promotes increased Ca and P absorption from the gut, increases Ca uptake by VSMCs, reduces VSMC migration and differentiation, anti-inflammatory effect, and negative regulation of the renin–angiotensin–aldosterone system[32]	[a]
Vitamin K	Inhibitor	Promotes γ-carboxylation of MGP and reduces calcification[20]	[a]

CKD, chronic kidney disease; NF-κB, nuclear factor-κB; VSMC, vascular smooth muscle cell.
[a]Not known.
[b]No effect seen in animal models.

to premature aging disorders such as arteriosclerosis, osteoporosis, and skin atrophy.[10,11] The vascular phenotype of these mice resembles the age-related disorder Mönckeberg's medial sclerosis in humans, with extensive medial calcification of all calibers of arteries. The bone system shows a low turnover osteoporosis, with decreased osteoblasts and osteoclasts, resembling senile osteoporosis.[10] *FGF23*-null mice have severe growth retardation, osteopenia, a markedly short life span, renal phosphate wasting, and also excessive mineralization in soft tissues with marked vascular calcification in the kidneys.[11] Additionally, both proteins can be detected in serum, but their role in the circulation has not been determined, and, so far, direct actions of Klotho and FGF23 on VSMC function have not been demonstrated.

NPP1

The enzyme NPP1 hydrolyzes ATP to generate inorganic pyrophosphate (PP_i), a physicochemical inhibitor of hydroxyapatite crystal growth. VSMCs constitutively express this calcification inhibitor within the normal aortic wall, and *NPP1*$^{-/-}$ mice develop medial calcification of the aorta with cartilaginous metaplasia, suggesting PP_i may act to inhibit this aberrant differentiation. The phenotype of animals is reminiscent of idiopathic infantile arterial calcification, linked to heritable NPP1 deficiency, and characterized by a severe and frequently lethal condition of widely disseminated arterial calcification with mineralization of the internal elastic lamina.[12] *In vitro* studies have shown that vascular damage induces the expression of alkaline phosphatase that inactivates PP_i locally and thus facilitates calcification.[36] However, PP_i is also found in the circulation and may exert its effects on many tissues via this conduit, although so far no experimental evidence exists to support this possibility. In hemodialysis patients PP_i levels are reduced,[26] and PP_i is also cleared by dialysis, suggesting that altered PP_i metabolism could contribute to vascular calcification in this group.

BMP signaling

BMPs are multifunctional cytokines originally identified as having diverse biological functions in bone remodeling and osteogenesis, but have since been found to play a role in multiple tissues. Some, including BMP7, which is necessary for normal kidney development, are also found at high levels in the circulation. Intracellular BMP signals are transduced by Smad proteins, with Smad6 and Smad7 acting as major negative regulators. Smad6 is not found in the circulation, but is expressed in the vasculature, predominantly in larger vessels, the same sites that exhibit cartilaginous metaplasia and ossification in the medial layer after targeted deletion of *Madh*6, the gene that encodes for Smad6.[8] Therefore, inhibitory Smad6 seems to limit the osteogenic responsiveness of cardiovascular cells to BMP signals to inhibit vascular calcification. Loss of Smad6 or an imbalance in local and circulating BMPs is thus likely to influence the progression of vascular calcification. This is important because circulating BMPs may be bound in serum by proteins such as MGP and thus a balance between circulating concentrations of a number of interacting proteins may be important for health. Indeed, administration of systemic BMP7 restores skeletal anabolic balance, reduces serum phosphate levels, and thereby reduces vascular calcification in experimental animal models of CKD. BMP7 is expressed primarily in the kidney and its expression levels are reduced in CKD.[37] Thus, further exploration of the role of this and other circulating BMPs in humans is likely to be informative.

CONCLUSIONS

The devastating effects of CKD on the vasculature are the net result of multiple pathogenic mechanisms that overwhelm natural defenses against calcification. Circulating toxic elements that cause VSMC damage and death accumulate while calcification inhibitors are decreased both locally and systemically. Studies in animal models have provided important insights into the pathogenesis of vascular calcification, but approaches to stop progression or even reverse the pathology are in their infancy. One key area for further investigation is the role of circulating proteins in regulating calcification, either by effects on maintenance of calcium phosphate homeostasis in solution or by direct effects on vascular cell function. It is likely that there are a number of as yet undiscovered circulating inhibitors that may be crucial in inhibiting calcification.

The relationship between vascular calcification and bone homeostasis is also crucial, as inhibitors may exert differential effects in these tissues. Moreover, some circulating inhibitors and activators such as osteocalcin and alkaline phosphatase are produced by bone, and their levels are used as indicators of bone turnover, but whether they exert effects on soft tissue calcification is unknown. Additionally, it will be crucial to determine whether circulating proteins are complexed with serum or other proteins that limit their function. More speculatively, it will be important to determine if circulating inhibitors are complexed with hydroxyapatite from bone or, more importantly, circulate in vesicles released from VSMCs. Other key questions such as the tissue origins of circulating proteins, the impact of perturbations in their circulating levels on VSMC function, and whether changes in levels can predict calcification, all need to be explored further. Importantly, mechanisms of calcification are likely to be similar in all soft tissues, and kidney calcification may be one mechanism promoting progression of kidney failure. Therefore it is tempting to speculate that it may be possible to measure and globally limit the susceptibility of a patient to calcify by directly influencing what's circulating.

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